Peptide N1 (SEQ ID NO: 27), Peptide N2 (SEQ ID NO: 28), Peptide N3 (SEQ ID NO: 29), Peptide N4 (SEQ ID NO: 30), Peptide N5 (SEQ ID NO: 31), Peptide N6 (SEQ ID NO: 32) and Peptide N7 (SEQ ID NO: 33) described in Table 1.

Please delete Figures 1 and 3 and substitute therefor new Figures 1 and 3, attached, containing SEQ ID NO. references.

Please insert the attached Sequence Listing after page 46 and renumber the Claims pages to begin with page 63.

<u>REMARKS</u>

In response to the Notice to File Missing Parts, a paper copy of the Sequence Listing is attached. Instructions for its incorporation into the specification is provided above.

Also transmitted herewith is a copy of the Sequence Listing in computer readable form. As required by 37 CFR 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d) Applicants' Agent hereby states that the content of the Sequence Listing in paper form and content of the computer readable form of the Sequence Listing are the same, and the submission includes no new matter.

Respectfully submitted,

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1. (Marked Up)   A method for enhancing the solubility of, and promoting the adoption of native folding conformation, of a protein or polypeptide expressed by recombinant DNA techniques in a host cell, the method comprising:

a)   providing a first nucleic acid sequence encoding a protein or polypeptide of interest, the protein or polypeptide being substantially insoluble, or biologically inactive, when expressed in a host cell by recombinant DNA techniques;

b)   providing a second nucleic acid sequence encoding a peptide extension having a net negative charge, the peptide T7A (SEQ ID NO: 20) of Table 1 being specifically excluded;

c)   fusing the second nucleic acid sequence to the first nucleic acid sequence in an expression vector such that a fusion protein encoded by the first and second nucleic acid sequences is expressed in the host cell following transformation of the host cell with the expression vector encoding the fusion protein, the peptide extension encoded by the second nucleic acid sequence being positioned at the carboxyl-terminus of the protein or polypeptide of interest;

d)   transforming the host cell with the expression vector encoding the fusion protein; and

e)   culturing the transformed host cells under conditions appropriate for the expression of the fusion protein.

13. (Marked Up)   The method of Claim 11, wherein the peptide extension is selected

from the group consisting of: Peptide T7C (SEQ ID NO: 5), Peptide T7B

(SEQ ID NO: 6), Peptide T7B1 (SEQ ID NO: 7), Peptide T7B2 (SEQ ID

NO: 8), Peptide T7B3 (SEQ ID NO: 9), Peptide T7B5 (SEQ ID NO: 11),

Peptide T7B6 (SEQ ID NO: 12), Peptide T7B7 (SEQ ID NO: 13), Peptide

T7B8 (SEQ ID NO: 14), Peptide T7B9 (SEQ ID NO: 15), Peptide T7B10

(SEQ ID NO: 16), Peptide T7B11 (SEQ ID NO: 17), Peptide T7B12 (SEQ

ID NO: 18), Peptide T7B13 (SEQ ID NO: 19), Peptide T7A1 (SEQ ID NO:

21), Peptide T7A2 (SEQ ID NO: 22), Peptide T7A3 (SEQ ID NO: 23),

10   Peptide T7A4 (SEQ ID NO: 24) and Peptide T7A5 (SEQ ID NO: 25).

25. (Marked Up)   The method of Claim 23, wherein the peptide extension is

selected from the group consisting of: Peptide N1 (SEQ ID NO: 27), Peptide

N2 (SEQ ID NO: 28), Peptide N3 (SEQ ID NO: 29), Peptide N4 (SEQ ID

NO: 30), Peptide N5 (SEQ ID NO: 31), Peptide N6 (SEQ ID NO: 32) and

Peptide N7 (SEQ ID NO: 33).

26. (Marked Up)   A method for enhancing the *in vitro* renaturation of a protein or

polypeptide expressed by recombinant DNA techniques in a host cell, a

substantial percentage of the expressed protein or polypeptide being localized

in inclusion bodies following expression in the host cell, the method

comprising:

a)   providing a first nucleic acid sequence encoding a protein or

polypeptide of interest;

b)   providing a second nucleic acid sequence encoding a peptide

extension having a net negative charge, the peptide T7A (SEQ ID

10   NO: 20) of Table 1 being specifically excluded;

c)   fusing the second nucleic acid sequence to the first nucleic acid

sequence in an expression vector such that a fusion protein encoded

by the first and second nucleic acid sequences is expressed in a host

cell following transformation of the host cell with the expression

vector encoding the fusion protein, the peptide extension encoded by

the second nucleic acid sequence being positioned at the carboxyl-

terminus of the protein or polypeptide of interest;

d)   transforming the host cell with the expression vector encoding the

fusion protein, under conditions appropriate for expression of the

20   fusion protein;

e)   isolating inclusion bodies from lysates of the host cell;

f)   contacting the isolated inclusion bodies with a denaturing solution

thereby solubilizing the fusion protein comprising the inclusion body;

and,

g)   suspending the solubilized fusion protein of step f) in a renaturation

buffer.

39. (Marked Up)   The method of Claim 37, wherein the peptide extension is selected

from the group consisting of: Peptide T7C (SEQ ID NO: 5), Peptide T7B

(SEQ ID NO: 6), Peptide T7B1 (SEQ ID NO: 7), Peptide T7B2 (SEQ ID

NO: 8), Peptide T7B3 (SEQ ID NO: 9), Peptide T7B5 (SEQ ID NO: 11),

Peptide T7B6 (SEQ ID NO: 12), Peptide T7B7 (SEQ ID NO: 13), Peptide

T7B8 (SEQ ID NO: 14), Peptide T7B9 (SEQ ID NO: 15), Peptide T7B10

(SEQ ID NO: 16), Peptide T7B11 (SEQ ID NO: 17), Peptide T7B12 (SEQ

ID NO: 18), Peptide T7B13 (SEQ ID NO: 19), Peptide T7A1 (SEQ ID NO:

21), Peptide T7A2 (SEQ ID NO: 22), Peptide T7A3 (SEQ ID NO: 23),

10   Peptide T7A4 (SEQ ID NO: 24) and Peptide T7A5 (SEQ ID NO: 25).

52. (Marked Up)   The method of Claim 50, wherein the peptide extension is selected

from the group consisting of: Peptide N1 (SEQ ID NO: 27), Peptide N2 (SEQ

ID NO: 28), Peptide N3 (SEQ ID NO: 29), Peptide N4 (SEQ ID NO: 30),

Peptide N5 (SEQ ID NO: 31), Peptide N6 (SEQ ID NO: 32) and Peptide N7

(SEQ ID NO: 33).

64. (Marked Up)   The expression vector of Claim 62, wherein the peptide extension is

selected from the group consisting of: Peptide T7C (SEQ ID NO: 5), Peptide

T7B (SEQ ID NO: 6), Peptide T7B1 (SEQ ID NO: 7), Peptide T7B2 (SEQ

ID NO: 8), Peptide T7B3 (SEQ ID NO: 9), Peptide T7B5 (SEQ ID NO: 11),

Peptide T7B6 (SEQ ID NO: 12), Peptide T7B7 (SEQ ID NO: 13), Peptide

T7B8 (SEQ ID NO: 14), Peptide T7B9 (SEQ ID NO: 15), Peptide T7B10

(SEQ ID NO: 16), Peptide T7B11 (SEQ ID NO: 17), Peptide T7B12 (SEQ

ID NO: 18), Peptide T7B13 (SEQ ID NO: 19), Peptide T7A1 (SEQ ID NO:

21), Peptide T7A2 (SEQ ID NO: 22), Peptide T7A3 (SEQ ID NO: 23),

10   Peptide T7A4 (SEQ ID NO: 24) and Peptide T7A5 (SEQ ID NO: 25).

76. (Marked Up)   The expression vector of Claim 74, wherein the peptide extension is

selected from the group consisting of: Peptide N1 (SEQ ID NO: 27), Peptide

N2 (SEQ ID NO: 28), Peptide N3 (SEQ ID NO: 29), Peptide N4 (SEQ ID

NO: 30), Peptide N5 (SEQ ID NO: 31), Peptide N6 (SEQ ID NO: 32) and

Peptide N7 (SEQ ID NO: 33).

80. (Marked Up)   A method for enhancing the solubility of, and promoting the

adoption of native folding conformation, of a protein or polypeptide

expressed by recombinant DNA techniques in a prokaryotic cell, the

method comprising:

    a)   providing a first nucleic acid sequence encoding a protein or

        polypeptide of interest, the protein or polypeptide being

        substantially insoluble, or biologically inactive, when expressed in

        a prokaryotic cell by recombinant DNA techniques;

    b)   providing a second nucleic acid sequence encoding a peptide

        extension having a net negative charge, the peptide T7A (SEQ ID

        NO: 20) of Table 1 being specifically excluded;

    c)   fusing the second nucleic acid sequence to the first nucleic acid

        sequence in a prokaryotic expression vector such that a fusion

        protein encoded by the first and second nucleic acid sequences is

        expressed in a prokaryotic cell following transformation of the

        prokaryotic cell with the prokaryotic expression vector encoding

        the fusion protein, the peptide extension encoded by the second

        nucleic acid sequence being positioned at the carboxyl-terminus of

        the protein or polypeptide of interest;

    d)   transforming the prokaryotic cell with the prokaryotic expression

        vector encoding the fusion protein; and

    e)   culturing the transformed prokaryotic cells under conditions

        appropriate for the expression of the fusion protein.

82. (Marked Up)   A method for enhancing the *in vitro* renaturation of a

protein or polypeptide expressed by recombinant DNA techniques in a

prokaryotic cell, a substantial percentage of the expressed protein or

polypeptide being localized in inclusion bodies following expression in

the prokaryotic cell, the method comprising:

a)   providing a first nucleic acid sequence encoding a protein or

        polypeptide of interest;

b)   providing a second nucleic acid sequence encoding a peptide

        extension having a net negative charge, the peptide T7A (SEQ ID

10   NO: 20) of Table 1 being specifically excluded;

c)   fusing the second nucleic acid sequence to the first nucleic acid

        sequence in a prokaryotic expression vector such that a fusion

        protein encoded by the first and second nucleic acid sequences is

        expressed in a prokaryotic cell following transformation of the

        prokaryotic cell with the prokaryotic expression vector encoding

        the fusion protein, the peptide extension encoded by the second

        nucleic acid sequence being positioned at the carboxyl-terminus of

        the protein or polypeptide of interest;

d)   transforming the prokaryotic cell with the prokaryotic expression

20   vector encoding the fusion protein, under conditions appropriate

        for expression of the fusion protein;

e)   isolating inclusion bodies from lysates of the prokaryotic cell;

f)   contacting the isolated inclusion bodies with a denaturing solution

thereby solubilizing the fusion protein comprising the inclusion

body; and,

g)   suspending the solubilized fusion protein of step f) in a

renaturation buffer.

84. (Marked Up)   An antibody which binds specifically to one or more

polypeptides selected from the group consisting of: Peptide T7C (SEQ ID

NO: 5), Peptide T7B (SEQ ID NO: 6), Peptide T7B1 (SEQ ID NO: 7),

Peptide T7B2 (SEQ ID NO: 8), Peptide T7B3 (SEQ ID NO: 9), Peptide

T7B5 (SEQ ID NO: 11), Peptide T7B6 (SEQ ID NO: 12), Peptide T7B7

(SEQ ID NO: 13), Peptide T7B8 (SEQ ID NO: 14), Peptide T7B9 (SEQ

ID NO: 15), Peptide T7B10 (SEQ ID NO: 16), Peptide T7B11 (SEQ ID

NO: 17), Peptide T7B12 (SEQ ID NO: 18), Peptide T7B13 (SEQ ID NO:

19), Peptide T7A1 (SEQ ID NO: 21), Peptide T7A2 (SEQ ID NO: 22),

10   Peptide T7A3 (SEQ ID NO: 23), Peptide T7A4 (SEQ ID NO: 24) and

Peptide T7A5 (SEQ ID NO: 25), Peptide N1 (SEQ ID NO: 27), Peptide

N2 (SEQ ID NO: 28), Peptide N3 (SEQ ID NO: 29), Peptide N4 (SEQ ID

NO: 30), Peptide N5 (SEQ ID NO: 31), Peptide N6 (SEQ ID NO: 32) and

Peptide N7 (SEQ ID NO: 33) Peptide T7A5, N1, N2, N3, N4, N5, N6, and

N7 described in Table 1.
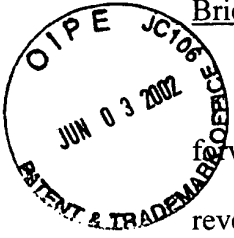
Brief Description of the Drawings, paragraphs 3, 4 and 6 (marked up)

Panel B: Illustrates the nucleic acid sequences (SEQ ID NOS: 34 and 36) of the forward and reverse PCR primers used to amplify CAR D1 (the complement of the reverse primer sequence is shown). Both primers were tailed with restriction sites (bold type) to facilitate cloning into the pET15b expression vector. Amino acid residues (SEQ ID NOS: 35 and 37) encoded by the primers are shown in single letter code.

Panel C: Illustrates the nucleotide sequences (SEQ ID NOS: 38 and 40) and amino acid sequences (SEQ ID NOS: 39 and 42) of the CAR D1-T7A fusion protein generated by ligation of the CAR D1 PCR product (shown in panel B) to the pET15b expression vector (both the PCR product and the pET15b plasmid were digested with NcoI and XhoI before ligation). The amino acid sequence of the resulting CAR D1-T7A fusion protein is shown in single letter code (SEQ ID NO: 41) on the top line (note that the central amino acid residues of CAR D1, from Ile 3 to Ala 125, are not shown, and are represented by .:.). The translation termination signal is indicated by *. Nucleotide sequences of restriction enzyme cleavage sites used to generate CAR D1-peptide fusion proteins are labeled and shown in bold type.

FIG. 3: Illustrates a schematic of the structure of vectors for fusion of a protein amino-terminus to peptide extensions. DNA fragments encoding the T7B peptide or various modified T7B peptides were amplified by PCR using primers that appended an upstream NcoI restriction site (SEQ ID NO: 43) and a downstream NdeI restriction site,site (SEQ ID NO: 44) encoding the amino acid sequences as shown in Panel A. The PCR products were then cloned between the NcoI and NdeI sites of pET15b, as shown in

Panel B. In the final ligated products, the 6-His tag (SEQ ID NO: 46) (which is normally present in pET15b) is replaced by the N-terminal peptides (SEQ ID NO: 45).

Description of the Invention, page 17, lines 3-13, (marked up)

A second nucleic acid sequence is provided which encodes a peptide extension having a net negative charge. The second nucleic acid is fused in-frame to the first nucleic acid in an expression vector such that a fusion protein encoded by the first and second nucleic sequences is expressed in the host cell following transformation of the host cell with the expression vector encoding the fusion protein. The peptide extension encoded by the second nucleic acid sequences is positioned at the carboxyl-terminus of the protein or peptide of interest. The peptide T7A (SEQ ID NO: 20) of Table 1 is specifically excluded in connection with this embodiment. In any jurisdiction which does not recognize a one-year grace period for filing a patent application following the public disclosure of an invention, it may also be necessary to exclude peptides T7B (SEQ ID NO: 6) and T7C (SEQ ID NO: 5) of Table 1 in connection with this and related embodiments.

Description of the Invention, page 19, lines 9-12, (marked up)

Examples of specific peptide extensions falling within the scope of the present invention include peptides T7C (SEQ ID NO: 5), T7B (SEQ ID NO: 6), T7B1 (SEQ ID NO: 7), T7B2 (SEQ ID NO: 8), T7B3 (SEQ ID NO: 9), T7B5 (SEQ ID NO: 11), T7B6 (SEQ ID NO: 12), T7B7 (SEQ ID NO: 13), T7B8 (SEQ ID NO: ~~T7C, T7B, T7B1,~~ ~~T7B2, T7B3, T7B5, T7B6, T7B7, T7B8, T7B9, T7B10, T7B11,~~ ~~T7B12, T7B13, T7A1, T7A2, T7A3, T7A4 and T7A5,~~ 14), T7B9 (SEQ ID

NO: 15), T7B10 (SEQ ID NO: 16), T7B11 (SEQ ID NO: 17), T7B12 (SEQ ID NO: 18), T7B13 (SEQ ID NO: 19), T7A1 (SEQ ID NO: 21), T7A2 (SEQ ID NO: 22), T7A3 (SEQ ID NO: 23), T7A4 (SEQ ID NO: 24) and T7A5 (SEQ ID NO: 25), as shown in Table 1.

Description of the Invention, page 20, lines 3-7, (marked up)

The specific amino-terminal peptide extensions exemplified comprise solubility or activity promoting portions of the 57 residue carboxyl-terminal portion of the T7 gene 10B protein, or variants thereof which result in the maintenance of a net charge ranging +2 to -20. Specifically, disclosed peptides include the following peptides which appear in Table 1: peptides N1, N2, N3, N4, N5, N6 and N7. N1 (SEQ ID NO: 27), N2 (SEQ ID NO: 28), N3 (SEQ ID NO: 29), N4 (SEQ ID NO: 30), N5 (SEQ ID NO: 31), N6 (SEQ ID NO: 32) and N7 (SEQ ID NO: 33).

Exemplification, sub-section II, lines 15-21, page 25 and lines 1-15, page 26 (marked up)

Expression of the CAR D1-T7A fusion protein (the sequence of the T7A peptide extension is shown in Table 1) (SEQ ID NO: 20) from the pET15b-CAR D1 construct was performed as follows. The pET15b-CAR D1 construct was transformed into *Escherichia coli* strain BL21-DE3 (Novagen, Inc.). Freshly transformed colonies were used to inoculate Luria-Bertani (LB) broth containing 150 mg/L penicillin G (Sigma), and the culture was grown at 37°C until mid-log phase (optical density approximately 0.8 at 600 nm). The culture was then chilled to 18°C and adjusted to 50 μM isopropyl D-thiogalactopyranoside (IPTG; Aldrich-Sigma) to induce protein expression. After incubation for an additional 5-20 hr at 18-20°C, the cells were harvested and analyzed for

expression of CAR D1. Cells were lysed by several cycles of rapid freezing and thawing in the presence of lysozyme, followed by sonic disruption with a probe tip sonicator (Heat Systems, Inc.). Lysates were then centrifuged, and the supernatant fraction was transferred to a fresh tube. Protein content in both the soluble (supernatant) and insoluble (pellet) fractions was examined by SDS-PAGE (electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulfate, a strong detergent and protein denaturant). Experimental results demonstrated that, when CAR D1 was fused to the 22 residue T7A peptide extension (SEQ ID No: 20), approximately 50% of the CAR D1 protein was present in the soluble fraction of cell lysates, whereas the remainder of the CAR D1 fusion protein was present in the insoluble pellet fraction (which contained the macroscopic inclusion bodies). In contrast, when the 22 residue peptide extension was eliminated by insertion of a stop codon upstream of the *Xho*I cloning site, the CAR D1 fusion protein was found to be completely aggregated into insoluble inclusion bodies (*See,* Freimuth, *et al.* (1999)).

Exemplification, sub-section III, lines 8-22, page 27 and lines 1-3, page 28 (marked up)

Additional experiments were performed in order to establish whether the mechanism of CAR D1 folding enhancement was specific for the T7A peptide (SEQ ID NO: 20) derived from the bacteriophage T7 gene 10B protein. The bacteriophage T7 gene 10 encodes two proteins, 10A and 10B, which are identical in amino acid sequence for the first 342 amino acid residues. Translation of the 10A protein is continued for three additional codons before terminating after codon 345, whereas a reading frame shift in codon 343 produces the 10B form which continues translation for a total of 56 additional codons before terminating after codon 398. *See,* Condron, *et al.,* J. Bacteriol.

173: 6998-7003 (1991). The sequence of the carboxyl-terminal 57 amino acid residues of

the bacteriophage T7 gene 10B protein (amino acid residues 343-398) is

~~FQSGVMLGVASTVAASPEEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEQ~~

FQSGVMLGVASTVAASPEEASVTSTEETLTPAQEAARTRAANKARKEAE

LAAATAEQ (SEQ ID NO: 1). The bacteriophage T7 gene 10A and 10B proteins are

structural proteins that form the icosahedral phage head. The unique 57 residue carboxyl-

terminus of the 10B protein is exposed on the surface of phage heads, but this peptide is

not essential for propagation of bacteriophage T7 under laboratory conditions. Indeed, in

the bacteriophage T7-based phage display system (see Novagen catalog and Studier, et al.

U.S. Patent No. 5,766,905), foreign peptides are substituted for the non-essential 10B C-

terminal 57 residue peptide, and thus become displayed on the phage head.

Exemplification, sub-section III, lines 10-20, page 28 (marked up)

To investigate the specificity of the T7A peptide-mediated folding of CAR D1,

the effects of bacteriophage T7 and T3 gene 10B-derived, carboxyl-terminal peptide

extensions on the folding of CAR D1 were compared. The DNA fragment encoding the

18 amino acid residue T7A peptide (SEQ ID NO: 20) was excised from the pET15b-CAR

D1 construct by digestion with restriction endonucleases *Bam*HI and *Blp*I (*see*, FIG. 1,

Panel C) and replaced with PCR products encoding either: (*i*) the complete 57 amino acid

residue T7 gene 10B terminal peptide (T7C) (SEQ ID NO: 5); (*ii*) a shorter fragment

encoding the terminal 40 amino acid residues of the T7 gene 10B terminal peptide (T7B)

(SEQ ID NO: 6); or (*iii*) a fragment encoding the terminal 39 amino acid residues of the

bacteriophage T3 gene 10B terminal peptide (T3) (SEQ ID NO: 26). These peptide

extensions were designated Peptide T7C (SEQ ID NO: 5), Peptide T7B (SEQ ID NO: 6),

and Peptide T3 (SEQ ID NO: 26). The amino acid sequences of these peptide extensions
are shown in Table 1.

Exemplification, sub-section IV, lines 6-19, page 30 (marked up)

A. Role of predicted amphipathic α-helices. Both the T7B and T7C peptides
were predicted by sequence analysis algorithms (*e.g.*, Chou/Fasman) to contain two long
α-helices, both of which have weak amphiphilic character as revealed by helical wheel
projections. It is conceivable that peptide extensions with weak amphiphilic character
could function as *cis*-acting chaperones by interacting transiently with hydrophobic
regions of the newly translated polypeptide to prevent aggregation. Accordingly, peptide
extension mutants were constructed to determine if amphiphilic α-helical character is
necessary for the protein folding activity of these peptides. Peptides T7B2 (SEQ ID NO:
8) and T7B3 (SEQ ID NO: 9) incorporate helix-disrupting proline or glycine residues at
the start of the predicted carboxyl-terminal helix, whereas Peptide T7B1 (SEQ ID NO: 7)
has a deletion that would disrupt the amphiphilic character of the predicted helix. None
of these three modified peptide extensions reduced the yield of soluble CAR D1
produced in *E. coli*. Thus, these results demonstrate that the folding activity of the T7B
and T7C peptide extensions does not depend on the ability of these peptides to form
amphiphilic α-helices.

Exemplification, sub-section IV, lines 16-22, page 32 and lines 1-2, page 33 (marked up)

The ssrA and T7 peptide extensions are similar in that both are carboxyl-terminal
modifications of their substrate proteins. Additionally, the T7 peptide contains a

sequence motif (AANKAR) (SEQ ID NO: 2) that is similar to the SspB recognition motif

in the ssrA peptide, AANDEN (SEQ ID NO: 3); where N is the dominant residue

recognized by SspB. However, unlike the ssrA tag, which is always fused to truncated

nascent polypeptides, the T7 peptides of the invention disclosed herein are fused to

complete, full-length proteins or protein domains. Therefore, if SspB and/or ClpX

recognize sequence elements in the T7 peptides, then these factors conceivably might

promote folding rather than degradation of intact proteins or protein domains.

Exemplification, sub-section IV, Lines 3-10, page 33 (marked up)

Accordingly, in order to determine whether the T7B peptide acts through a

mechanism that is dependent upon binding by SspB and/or ClpX, additional mutants

were constructed in which critical residues of the putative recognition sites for either

SspB (i.e., Peptide T7B11 (SEQ ID NO: 17) and Peptide T7B12 (SEQ ID NO: 18)) or

ClpX (i.e., Peptide T7B9 (SEQ ID NO: 15) and Peptide T7B10 (SEQ ID NO: 16)) were

altered or deleted. Experimental results demonstrated that the yield of soluble CAR D1

was not reduced by any of these aforementioned mutations, indicating that these *trans-*

acting factors do not contribute to the mechanism of T7B-mediated folding of CAR D1.

Exemplification, sub-section IV, lines 11-21, page 33 and lines 1-8, page 34 (marked up)

C.   **Role of peptide net charge.** During analysis of T7 peptide mutants

generated for the studies described above, it was observed that the partial folding-activity

of peptide T7A (SEQ ID NO: 20) was increased by mutation to peptide T7A1 (SEQ ID

NO: 21), and, conversely, that the full folding-activity of peptide T7B (SEQ ID NO: 6)

was reduced by mutation to peptide T7B4 (SEQ ID NO: 10). The T7A1 (SEQ ID NO: 21) mutant was constructed to disrupt the weak amphiphilic character of the peptide, whereas a T7B4 (SEQ ID NO: 10) mutant was constructed to probe the length-dependence of the folding activity. However, as may be ascertained from Table 1, the mutation in Peptide T7A1 (SEQ ID NO: 21) increases the peptide net charge from -3 to -4, whereas the Peptide T7B4 (SEQ ID NO: 10) mutation decreases the peptide net charge from -6 to -2. Based on these results, additional mutants were constructed in order to systematically examine whether there was a correlation between peptide net charge and ability to mediate folding of CAR D1. As demonstrated by the experimental results not shown here, the relative proportion of soluble CAR D1 produced in *E. coli* increased as the net negative charge on Peptide T7A (SEQ ID NO: 20) was increased from -3 to -6 (peptides ~~T7A1, T7A2, and T7A3~~ T7A1 (SEQ ID NO: 21), T7A2 (SEQ ID NO: 22), and T7A3 (SEQ ID NO: 23)). Both Peptides T7A3 (SEQ ID NO: 23) and T7B (SEQ ID NO: 6) were found to produce almost a 100% yield of soluble CAR D1, and both species had a net negative charge of -6. Therefore, the characteristic of the carboxyl-terminal peptide extensions that is critical for their ability to mediate folding of CAR D1 appears to be the size of the net negative charge carried by the peptide extension. Consistent with this conclusion, the T3 peptide (SEQ ID NO: 26) extension, which is unable to fold CAR D1, has a net charge of -2.

Exemplification, sub-section V, lines 3-17, page 35 (marked up)

  In order to distinguish between these two possible mechanisms, the effect of peptide extensions on the folding of other test proteins was examined. In one experiment,

the distal domain of the human A33 protein (Heath, et al., Proc. Natl. Acad. Sci. U.S.A.

94: 469-474 (1997)), the protein that is most similar to CAR D1 as revealed by homology

searching using the BLAST-P program (32% identical), was examined. A33 and CAR

are both members of the immunoglobulin superfamily and have similar protein and gene

organization. See, Chretien, et al., Eur. J. Imunol. **28**: 4094-4104 (1998). A cDNA

fragment encoding the A33 distal domain (D1) was amplified by PCR and cloned into the

pET15b-T7A construct in the same manner as schematically illustrated in FIG. 1 for

CAR D1. When a stop codon was included to prevent fusion to the T7 peptide, the A33

protein was found to be insoluble, as was also found for CAR D1. However, unlike the

results obtained with CAR D1, extending the carboxyl-terminus of A33 D1 with the T7B

peptide (SEQ ID NO: 6) did not increase A33 D1 solubility. Therefore, the T7B peptide

(SEQ ID NO: 6) does not appear to universally promote protein folding in vivo,

supporting the conclusion that these peptides do not function by recruiting chaperones to

the misfolded protein.

Exemplification, sub-section V., lines 18-21, page 35 and lines 1-18, page 36 (marked
up)

       To determine if further increasing the peptide extension net negative charge

would enhance folding of A33 D1, the A33 D1 domain was fused to Peptide T7B7 (SEQ

ID NO: 13), which has a net charge of - 12 (see, Table 1). Results demonstrated that the

A33 D1-T7B7 fusion protein was distributed approximately equally between the soluble

and insoluble fractions of cell lysates. Only a slight further increase in fusion protein

solubility resulted when A33 D1 was fused to Peptide T7B8 (SEQ ID NO: 14) (data not

shown), which has a net charge of - 16 (see, Table 1). Because the function of A33 is

unknown and consequently there is no assay for its biological activity, the A33 D1-T7B7

conformation was characterized by limited proteolysis. Staphylococcal V8 protease

digested the T7B7 (SEQ ID NO: 13) peptide extension more readily than the A33 D1

domain itself, as was observed for CAR D1 fusion proteins, generating digestion products

which migrated with slightly faster mobility than the intact protein in~~SDS~~ SDS-PAGE.

However, unlike CAR D1, the A33 D1 domain and the T7B7 (SEQ ID NO: 13) peptide

extension were equally sensitive to digestion with trypsin. Thus, although the A33 D1-

T7B7 fusion protein is soluble, it may have a non-native conformation. This was further

supported by the observation that the A33 D1-T7B7 fusion protein resolves into several

species with distinct mobilities when electrophoresed under non-denaturing conditions.

Together these results suggested that although the carboxyl-terminal peptide extension

was able to partially solubilize A33 D1, it may not be able to mediate proper folding of

the domain. Concomitant control experiments showed that both peptides T7B7 (SEQ ID

NO: 13) and T7B8 (SEQ ID NO: 14) promote folding of CAR D1 into its biologically

active conformation (data not shown), indicating that these peptides are compatible with

in vivo folding of at least some proteins.

Exemplification, sub-section V., lines 19-21, page 36 and lines 1-12, page 37 (marked up)

The analysis was extended to determine if the folding of other proteins could be

enhanced *in vivo* by extending the protein C-terminus with the T7B peptide (SEQ ID NO:

6) and more highly charged derivatives (T7B5-T7B8) (SEQ ID NOS: 11, 12, 13, 14,

respectively). The E. coli ClpX protein, a ~50 kD chaperone, misfolds and aggregates

into inclusion bodies when overexpressed in E. coli using pET vector technology. ClpX,

therefore, is an example of how the conditions of protein overexpression can render E.

coli unable to properly fold even its own endogenous proteins. As discussed above, this

may result from a deficit of one or more chaperones that are required to fold nascent

polypeptide chains. Fusion of the ClpX C-terminus to T7B (SEQ ID NO: 6) or to T7B5-

T7B8 (SEQ ID NOS: 11-14, respectively) peptides increased the fraction of the protein

that was recovered in the soluble fraction of cell lysates. However, in contrast to the

results obtained with A33, the C-terminal peptide extensions could be readily cleaved

from the ClpX protein by limited proteolysis with both trypsin and V8 protease.

Furthermore, after proteolytic removal of the T7B (SEQ ID NO: 6) C-terminal extension,

the resulting processed ClpX protein had full biological activity both in terms of ATPase

activity and ability to cooperate with the ClpP proteasome in degrading model protein

substrates.

Exemplification, sub-section V., lines 13-19, page 37 (marked up)

A group of thirteen yeast proteins which are known to form inclusion bodies

when over-expressed in E. coli using pET expression vectors were separately fused to the

T7B (SEQ ID NO: 6) peptide extension. Solubility and folding of six of these proteins

was rescued to greater than 50%, while another two were rescued to a lesser extent.

Solubility and folding of the remaining five proteins was not measurably affected by the

T7B peptide (SEQ ID NO: 6) extension (Table 2). Fusion to C-terminal peptide T7B7

(SEQ ID NO: 13) failed to increase the solubility of these five refractory yeast proteins.

Exemplification, sub-section VI., lines 17-20, page 39 and 1-2, page 40 (marked up)

This model was tested by fusing the CAR D1 N-terminus to amino-terminal peptide extensions, according to the method outlined in Figure 3. Consistent with the above-stated model, CAR D1 was least soluble when fused to the N-terminal peptide extensions N2 (SEQ ID NO: 28) and N3 (SEQ ID NO: 29) (which have neutral or +1 net charges, respectively). By contrast, CAR D1 was mostly soluble when fused to the N-terminal peptides N1 (SEQ ID NO: 27) and N4 (SEQ ID NO: 30), which have net charges of -2 and +2, respectively.

Exemplification, sub-section VI., lines 3-11, page 40 (marked up)

Results of further testing with other protein substrates were not completely consistent with this model, however. For example, the solubility of the 50 kD ClpX protein was significantly increased by fusion to the N-terminal peptide extension N2. Because the N2 peptide (SEQ ID NO: 28) has no net charge, it seems unlikely that this peptide could rescue of the folding of ClpX by a mechanism dependent on peptide net charge. Rather, in this case the N-terminal peptide extension may alter the initial folding pathway of the nascent polypeptide, fortuitously avoiding the formation of folding intermediates that may precipitate or be minimally soluble under conditions of chaperone deficit. Alternatively, the N-terminal peptides may recruit chaperones to the nascent polypeptide chain.

Exemplification, sub-section VII., lines 13-21, page 40 and lines 1-5, page 41 (marked up)

During *in vitro* refolding of denatured proteins, precipitation and aggregation of the protein upon removal of the denaturing agent is a common side reaction. Thus, precipitation and aggregation are problematic side reactions during the folding of proteins both *in vivo* and during refolding *in vitro*. Since carboxyl-terminal peptide extensions which carry a large net negative charge inhibit protein aggregation *in vivo*, possibly by increasing electrostatic charge repulsion between nascent polypeptide chains, experiments were performed to investigate whether such peptide extensions could inhibit protein aggregation during protein refolding reactions *in vitro*. To test this hypothesis, the A33 D1 protein fragment was produced in 2 different forms, with or without a T7B6 peptide (SEQ ID NO: 12) carboxyl-terminal extension. Both forms of the A33 D1 protein were produced with an amino-terminal 6-histidine tag. When protein expression was induced at 37°C, both A33 D1 and A33D1 D1-T7B6 proteins misfolded and accumulated in inclusion bodies (note that A33 D1-T7B6 is only partially soluble when induction is carried out at temperatures below 25°C).

Exemplification, sub-section VIII., lines 20-21, page 42 and lines 1-4, page 43 (marked up)

A synthetic peptide corresponding in sequence to peptide T7A was produced, as shown:

(acetyl-cysteine)-LEDPAANKARKEAELAAATAEQ (SEQ ID NO: 4).

An amino-terminal cysteine residue was incorporated into the peptide to introduce a reactive sulfhydryl group which could be utilized to couple the peptide to solid supports or carrier proteins.

Exemplification, Table I, page 45 (marked up)

Table 1

Peptide Name	Sequence	Net Charge[a]	SEQ ID NO
T7C	*LED*PFQSGVMLGVASTVAASPEEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEQ	−6	5
T7B	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEQ	−6	6
T7B1	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANKARKEAEL---TAEQ	−6	7
T7B2	*LEDP*-----------------EEASVTSTEETLTPAQEAARTR**PP**NKARKEAELAAATAEQ	−6	8
T7B3	*LEDP*-----------------EEASVTSTEETLTPAQEAARTR**GG**NKARKEAELAAATAEQ	−6	9
T7B4	*LEDP*--------------------------------TPAQEAARTRAANKARKEAELAAATAEQ	−2	10
T7B5	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANKARKEAEL**EAE**TAEQ	−8	11
T7B6	*LEDP*-----------------EEASVTSTEETLTPAQEAA**ETE**AANKARKEAEL**EAE**TAEQ	−12	12
T7B7	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANKA**EEE**AEL**EAE**TAEQ	−12	13
T7B8	*LEDP*-----------------EEASVTSTEETLTPAQEAA**ETE**AANKA**EEE**AEL**EAE**TAEQ	−16	14
T7B9	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANKARKEAELAA-----	−5	15
T7B10	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANKARKEAELAAA----	−5	16
T7B11	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAA**A**KARKEAELAAATAEQ	−6	17
T7B12	*LEDP*-----------------EEASVTSTEETLTPAQEAARTR---KARKEAELAAATAEQ	−6	18
T7B13	*LEDP*-----------------EEASVTSTEETLTPAQEAARTRAANK---EAELAAATAEQ	−8	19
T7A	*LEDP*--AANKARKEAELAAATAEQ	−3	20
T7A1	*LEDP*--**ERN**KERKEAELAAATAEQ	−4	21
T7A2	*LEDP*--**ERN**KERKEAEL**EAA**TAEQ	−5	22
T7A3	*LEDP*--**ERN**KERKEAEL**EAE**TAEQ	−6	23
T7A4	*LEDP*--AANKARKEAEL**EAA**TAEQ	−4	24
T7A5	*LEDP*--AANKARKEAEL**EAE**TAEQ	−6	25
T3	*LEDP*-----------------AVWEAGKVVAKGVGTADITATTSNGLIASCKVIVNAATS	−2	26
N1	M-EEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEH	−2	27
N2	MAE**RA**SVTSTEETLTPAQEAARTRAANKARKEAELAAATAEH	0	28
N3	MAE**EAK**VTSTEETLTPAQEAARTRAANKARKEAELAAATAEH	+1	29
N4	MAE**RAKR**TSTEETLTPAQEAARTRAANKARKEAELAAATAEH	+2	30
N5	M-EEASVTSTEETLTPAQEAARTRAANKARKEAEL**EAE**TAEH	−4	31
N6	M-EEASVTSTEETLTPAQEAA**ETE**AANKARKEAEL**EAE**TAEH	−8	32
N7	M-EEASVTSTEETLTPAQEAARTRAANKA**EEE**AEL**EAE**TAEH	−8	33

[a]The terminal COO⁻ and NH$_3^+$ groups of carboxyl-terminal and amino terminal peptide extensions were included in the calculation of peptide net charge